FORM PTO-1390 (Modified) (REV 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: 294-120 PCT/US

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR): 10/049473

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/NL00/00569	August 14, 2000	August 13, 1999

TITLE OF INVENTION
PNEUMOCOCCAL VACCINES

APPLICANT(S) FOR DO/EO/US
de Groot, Ronald, et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include itens (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☒ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

Copy of the international patent application as published under International Publication Number WO 01/12219 A1

EXPRESS MAIL CERTIFICATE

Date: 2-12-02 Label No. EL709114327US

I hereby certify that the date indicated above, I deposited this paper or fee with the U.S. Postal Service and that it was addressed for delivery to the Commissioner for Patents, Washington, DC 20231, by "EXPRESS MAIL Post Office to Addressee" Service.

Joan Newbert (Print Name) Joan Newbert (Signature)

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
10/049473	PCT/NL00/00569	294-120 PCT/US

24. The following fees are submitted:.

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

		CALCULATIONS PTO USE ONLY
☐	Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1040.00**	
☒	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**	
☐	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$740.00**	
☐	International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**	
☐	International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**	
	ENTER APPROPRIATE BASIC FEE AMOUNT =	**$890.00**
	Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	**$0.00**

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	21 - 20 =	1	x $18.00	**$18.00**
Independent claims	12 - 3 =	9	x $84.00	**$756.00**
Multiple Dependent Claims **(check if applicable)**			☐	**$0.00**

TOTAL OF ABOVE CALCULATIONS =	**$1,664.00**
☐ Applicant claims small entity status. See 37 CFR 1.27). The fees indicated above are reduced by 1/2.	**$0.00**
SUBTOTAL =	**$1,664.00**
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +	**$0.00**
TOTAL NATIONAL FEE =	**$1,664.00**
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☐	**$0.00**
TOTAL FEES ENCLOSED =	**$1,664.00**
Amount to be: refunded	$
charged	$

a. ☒ A check in the amount of **$1,664.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **08-2461** A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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SIGNATURE

Ronald J. Baron
NAME

29,281
REGISTRATION NUMBER

February 12, 2002
DATE

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s)	de Groot, et al.	Examiner:	Unassigned
Serial No:	Unassigned	Group Art Unit:	Unassigned
Confirmation No:	Unassigned	Docket:	294-120 PCT/US
Filed:	Herewith	Dated:	February 12, 2002
For:	PNEUMOCOCCAL VACCINES		

Commissioner for Patents
Washington, DC 20231

Express Mail Certificate:
Date: February 12, 2002 Label No: **EL709114327US**
I hereby certify that on the date indicated above, I deposited this paper or fee with the United States Postal Service and that if was addressed for delivery to the Assistant Commissioner for Patents, Washington, D.C. 20231 on **February 12, 2002**
Signature: Joan M. Newbert

PRELIMINARY AMENDMENT

Sir:

In order to place the present application in condition for examination on the merits Applicant submits the following amendment for entry in the above-identified application.

IN THE SPECIFICATION:

On page 1, before line 1, after the title, please insert the following:

This application is the U.S. National Phase of International Application Number PCT/NL00/00569, filed August 14, 2000, which is incorporated herein by reference.

BACKGROUND OF THE INVENTION

On page 2, after line 22, insert the following:

SUMMARY OF THE INVENTION

On page 3, after line 13, insert the following:

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS IN THE CLAIMS:

IN THE CLAIMS:

Please amend Claims 3 - 8, 14, and 15, to read as follows:

3. (Amended) The vaccine or medical preparation according to claim 1, further comprising a suitable adjuvant or carrier.

4. (Amended) The vaccine or medical preparation according to claim 1, wherein said protein comprises an amino acid sequence as shown in fig. 1B.

5. (Amended) The vaccine or medical according to claim 1, wherein said protein is the protein maturation protein from *S. pneumoniae* Ft231 or EF3296.

6. (Amended) The vaccine or medical preparation according to claim 1, wherein said fragment comprises an anchoring fragment, an antigenic fragment or a functional equivalent thereof for a functional equivalent of a receptor binding site or an antibody binding site.

7. (Amended) The vaccine or medical preparation according to claim 1, wherein said protein or said fragment comprises a purified, partly purified, recombinant or synthetic protein or fragment thereof.

8. (Amended) The vaccine or medical preparation according to claim 1, wherein said fragment comprises at least 8 amino acids.

14. (Amended) Method of treatment of *S. pneumoniae* infection comprising administering a vaccine according to claim 1.

15. (Amended) Method for the vaccination of a mammal against an infection of *S. pneumoniae* comprising administering a suitable dose of a vaccine according to claim 1.

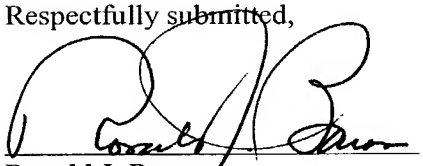
REMARKS

In order to place the present application in condition for examination in the U.S. Patent Office, Applicant has amended the Specification and Claims to conform to U.S. practice. No new subject matter has been introduced as a result of this Amendment. As a

result of the present Amendment, Claims 1-21 remain in the application for purpose of prosecution.

As a result of this Amendment no additional fees should be assessed as a result of filing multiple dependent claims. Therefore, since new matter has not been introduced as a result of this Amendment, entry hereof and examination and favorable consideration are respectfully requested. Any questions regarding this matter should be directed to the undersigned.

Respectfully submitted,



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VERSION OF AMENDMENT WITH MARKS TO SHOW CHANGES MADE

IN THE SPECIFICATION:

On page 1, before line 1, after the title, please insert the following:

This application is the U.S. National Phase of International Application Number PCT/NL00/00569, filed August 14, 2000, which is incorporated herein by reference.

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On page 2, after line 22, insert the following:

SUMMARY OF THE INVENTION

On page 3, after line 13, insert the following:

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS IN THE CLAIMS:

IN THE CLAIMS:

Please amend Claims 3 - 8, 14, and 15, to read as follows:

3. (Amended) The vaccine or medical preparation according to claim 1 [or 2], further comprising a suitable adjuvant or carrier.

4. (Amended) The vaccine or medical preparation according to claim 1 [anyone of the claims 1-3], wherein said protein comprises an amino acid sequence as shown in fig. 1B.

5. (Amended) The vaccine or medical according to claim 1 [anyone of the claims 1-4], wherein said protein is the protein maturation protein from *S. pneumoniae* Ft231 or EF3296.

6. (Amended) The vaccine or medical preparation according to claim 1 [anyone of the claims 1-5], wherein said fragment comprises an anchoring fragment, an antigenic fragment or a functional equivalent thereof for a functional equivalent of a receptor binding site or an antibody binding site.

7. (Amended) The vaccine or medical preparation according to claim 1 [anyone of the claims 1-6], wherein said protein or said fragment comprises a purified, partly purified, recombinant or synthetic protein or fragment thereof.

8. (Amended) The vaccine or medical preparation according to claim 1 [anyone of the claims 1-7], wherein said fragment comprises at least 8 amino acids.

14. (Amended) Method of treatment of *S. pneumoniae* infection comprising administering a vaccine according to claim 1 [claims 1-8].

15. (Amended) Method for the vaccination of a mammal against an infection of *S. pneumoniae* comprising administering a suitable dose of a vaccine according to claim 1 [anyone of the claims 1-8].

2/Ptrs

Title: Pneumococcal vaccines.

The invention relates to the field of vaccines against microbial infections and especially bacterial vaccines, in particular to pneumococcal vaccines.

Streptococcus pneumoniae (pneumococcus, *S. pneumoniae*) is an important pathogen, which causes significant morbidity and mortality throughout the world. *S. pneumoniae* is a major cause of invasive diseases such as meningitis, bacteremia, and pneumonia, as well as non-invasive diseases like acute otitis media and sinusitis (1). In young children, the pneumococcus is often part of the normal nasopharyngeal flora. Especially during the first two years of life, children are colonised with novel strains of pneumococci. Children colonised with *S. pneumoniae* develop more often acute otitis media than children who are not colonised (2, 3, 4).

The precise molecular mechanisms through which the pneumococcus invades and damages host tissues are not fully understood. For many years, the polysaccharide capsule has been recognised in the art as the major virulence factor and, consequently, an important vaccine candidate (for review, see 5, 6). The current pneumococcal vaccine strategies focus on the use of conjugates, in which a limited number of different capsular polysaccharides are linked to a carrier protein (7,8). Although the results of early trials look promising, problems still arise since large-scale vaccination over time generally leads to a shift in serotype distribution towards capsular types that are poorly immunogenic or not included in the vaccine. Such a shift may be enhanced by the frequent horizontal exchange of capsular genes, as described by several investigators (9, 10, 11).

Over the last few years, much attention has been focused on the role of pneumococcal proteins in pathogenesis and protection. Proteins that are involved in the pathogenesis of infections by *S. pneumoniae* are considered to be interesting components for future conjugate vaccines. Such proteins are able to switch the immune response against the polysaccharides present in the vaccine from T-cell independent to T-cell dependent, through which the antibody response towards the polysaccharides may be increased and a memory response will be provided. In addition, such proteins should provide protection against colonisation and infection

with *S. pneumoniae* strains whose capsular polysaccharides are not included in the vaccine.

The protective abilities of various (virulence) proteins have been investigated previously. Immunisation of pneumolysin (12), pneumococcal surface protein A (PspA) (13, 14,15) pneumococcal surface adhesin A (PsaA) (16), and neuraminidase (17) clearly confer protection in animals.

In the literature various polynucleotides of *S. pneumoniae* and polypeptides predicted to be encoded by said nucleotides have been reported and the use of these compounds in vaccines and medicinal preparation has been contemplated, for instance in WO 97/37026 and WO 98/18930. These publications however, do not identify any functional protein let alone a vaccine based on a functional protein. These publications are further silent in respect of proteins that when used in vaccines are able to elicit an immuneresponse let alone that they are able to elicit any protective, more in particular opsonophagocytic activity.

The publications further do not disclose any information regarding cross reactivity towards various strains of *S. pneumoniae* in a relevant vertebrate host. Furthermore these publications do not describe the protease maturation protein of *S. pneumoniae*. Another publication that relates to the present invention is WO 00/06737. This publication discloses a pool of several hundreds of proteins. Most of these proteins, including the protein described in the present invention have not been tested for their immuneresponsive properties, opsonophagocytic activity or cross reactivity.

The present invention identifies surface-associated proteins from *S. pneumoniae* with immune-protective properties, more in particular opsonophagocytic activity. Furthermore the present invention provides the use of these proteins as vaccine components and their use in conjugate vaccination strategies. The invention further provides for antibodies which express opsonophagocytic activity and methods for their production, as for example detailed in the experimental part.

It has now been found that a surface-associated protein of *S. pneumoniae* can be used in the preparation of a vaccine against micro-organisms and especially *S. pneumoniae*. This surface protein is present in a large number of strains of *S. pneumoniae*.

The invention accordingly relates to a vaccine or medical preparation comprising a protease maturation protein of S. pneumoniae and/or a fragment thereof and/or a homologous and/or functionally homologous protein and/or fragment thereof for the treatment of microbial infections and especially of *S. pneumoniae* infections and for the generation of antibodies in an immunised or vaccinated in a vertebrate host and which expresses opsonophagocytic activity against *S. pneumoniae* and infections thereof. The invention also relates to the use of protease maturation protein of *S. pneumoniae* or a fragment thereof for the preparation of a vaccine for the treatment of a *S. pneumoniae* infection and/or colonisation and to the use of a protease maturation protein of *S. pneumoniae* or a fragment thereof or a recombinant or synthetic protein or fragment or functionally homologous protein or fragment thereof as a carrier for inducing prophylactic protection against other micro-organisms including viruses.

In this description and the appending claims treatment encompasses and generally is the prophylaxis of infections.

Surface-associated proteins were isolated or purified from the *S. pneumoniae* strains FT231 and EF3296, respectively, using either the SB14 extraction procedure or the Triton X114 extraction procedure as further illustrated in the working examples herein-below. The proteins and polypeptides were purified in relatively high concentrations, as shown by two-dimensional SDS-PAGE. Extracts from either strain resulted in a highly homologous protein profile as demonstrated by computer-assisted analysis. Since both extraction procedures resulted in comparable protein profiles, the SB14 extraction procedure was used for further experiments.

Hyperimmune serum antibodies were raised against the pneumococcal surface-associated proteins of *S. pneumoniae* strains FT231 and EF3296, respectively. To confirm the presence of surface-exposed proteins in the fraction, the sera were tested for the recognition of components at the surface of pneumococcal whole cells. Immuno-cytometric experiments demonstrated the recognition of components exposed at the surface of the homologous pneumococcal strains by the hyperimmune sera. Heterologous immuno-cytometric analysis demonstrated that the serum-recognition of components at the surface of the two strains display partial overlap as the level of fluorescence of the bacteria using the homologous serum was greater than the fluorescence level using the heterologous serum. In addition,

components at the surface of eleven other pneumococcal strains, which display ten distinct genotypes and represent eight clinically important serotypes, were invariably recognised by the hyperimmune sera. The strains which have tested are described in more epidemiological detail by Hermans et al. (10).

Hyperimmune rabbit sera raised against the surface-associated pneumococcal proteins in the phagocytosis assay as described by Alonso Develasco et al. (5) have been analysed. The *in-vitro* opsonophagocytic activity of the serum is presumed to correlate with *in-vivo* protection against *S. pneumoniae*. The opsonophagocytic activity of the hyperimmune sera was high using the homologous pneumococcal strains. The specificity of the serum opsonophagocytic activity was determined using seven genotypically distinct pneumococcal strains, representing seven serotypes that cause most infections in young children and two strains of the genetically closely related species *S. bovis* and *Enterococcus faecalis*, respectively. The hyperimmune rabbit sera were invariably opsonically active against the pneumococcal strains. In contrast, the serum opsonophagocytic activity was very low using *S. bovis* and *E. faecalis*. This means that *S. bovis* and *E. faecalis* are not recognised by the serum. Apparently these organisms have insufficient homology to *S. pneumoniae* for serological recognition.

All immunodominant proteins were cut from two-dimensional acrylamide gels. Protein characterisation was performed using mass spectrometric analysis (Maldi-tof) to analyse trypsine fragments on the amino acid level. In addition, monospecific hyperimmune rabbit serum antibodies were raised against the acrylamide-embedded proteins. The monospecific hyper-immune sera were used to identify the cellular localisation of the proteins by immuno-electron microscopy and to determine the capacity of these proteins to elicit opsono-phagocytosis.

Blast and/or Blastp computer programs were used for comparison of the sequence of the protein isolated from *S. pneumoniae* with known sequences in various databases. In this program the Expect value (E-value) is a parameter that describes the number of hits that can be expected just by chance when searching a database. The E value is a measurement for the random background noise that exists from a match between two sequences. To decide whether or nbot a protein is functionally homologous with Pmp, a homology cut-off value is defined as an E-value of 10^{-10}. A

protein with an E-value of more than 10^{-10} is not considered sufficient homologous to Pmp from *S. pneumoniae*.

One of the proteins revealed to be homologous to a polypeptide encoded by nucleotide sequence 7632-8597 on contig 33 of *S. pneumoniae* (Figure 1). This ORF was identical to ORF 414 of *S. pneumoniae* in the WIT-system. Details about the WIT system can be found on http://wit.mcs.anl.gov/ and on the website of The Institute for Genomic Research, Rockville USA updated on April 7, 1999.

Since this pneumococcal polypeptide was related to protease maturation protein *Lactobacillus paracasei* (Swiss Prot acc. nr. Q02473) (Figure 2), and *Lactococcus lactis subspec. lactis* (Swiss Prot acc. nr. P15294) (Figure 3) and *Lactococcus lactis subsp. cremoris* (Swiss Prot acc. nr. P14308) (Figure 4) it was designated the protease maturation protein (Pmp) of *S. pneumoniae*. Also the molecular weight of the protein cut from the acrylamide gel corresponds with the molecular weight of Pmp.

This protein has various interesting characteristics with respect to its use in conjugate vaccines.

The immuno-electron microscopy using the monospecific rabbit antibodies raised against Pmp demonstrated that this protein was surface-associated.

The opsonophagocytic activity of the monospecific anti-Pmp rabbit antibodies was measured using the homologous pneumococcal strain, as well as seven genotypically distinct pneumococcal strains, representing seven serotypes that causes most infections in young children and two strains of the genetically closely related species *S. bovis* and *E. faecalis*, respectively. The anti-Pmp rabbit antibodies were invariably opsonically active against the pneumococcal strains. In contrast, the serum opsonophagocytic activity was very low using *S. bovis* and *E. faecalis*. These data show that Pmp has the ability to elicit immune protection, which is a major requisite with respect to its use as a vaccine component. Thus, not only the existence of the protein has been demonstrated, also its potential function and properties have been adequately established, which distinguishes the present invention over the art.

DNA sequence analysis of the *Pmp* genes of the homologous pneumococcal strain, as well as fifteen genotypically distinct pneumococcal strains, representing fourteen serotypes that cause most infections in young children demonstrated very limited variation. This is an important feature of Pmp with respect to its use as a

vaccine component, and of the present invention in general, as it will guarantee immunological cross reactivity.

Phenotypic variation is an important mechanism allowing bacterial pathogens to adapt to different host environments. In *S. pneumoniae*, phenotypic variation due to alterations in cell-surface structures can be detected as spontaneous, reversible changes in colony morphology. Such alterations result in opaque and transparent colonies within single strains. The relationship of several previously identified cell-surface structures to phenotypic variation has recently been described (18). The transparent phenotype incorporates significantly more surface-exposed phosphorylcholine. In addition, the expression of three choline-binding proteins (Cbp) also varies in the phenotypic variants. The expression of autolysin LytA, is lower in opaque variants as compared to transparent variants, pneumococcal surface protein PspA is present in higher amounts in opaque variants, and CbpA is present in higher amounts in transparent variants. Such phenotypic changes also result in alterations in virulence phenotype. The opaque phenotype has decreased ability to colonise the nasopharynx as compared to the transparent phenotype (19). In addition, the survival time of mice after intraperitoneal challenge of the opaque phenotype is decreased as compared to the transparent phenotype (20)

Pmp is predominantly present in transparent colony variants of *S. pneumoniae*. Since these variants are prone to colonise the nasopharynx in animal models (21), immunisation with conjugate vaccines containing Pmp or Pmp components will enhance the removal of colonising pneumococci from the nasopharynx.

The determination of the function of Pmp in *S. pneumoniae* has been based on the homology of the protein with Pmp proteins of other bacterial species. The function of the Pmp proteins of other bacterial species is generally the activation of certain proteases. The most important keys to the use of Pmp in vaccines is the surface exposure of Pmp, whereby Pmp is available to the immunesystem and the elicitation of opsonophagocytic activity as shown in the opsonophagocytosis assay.

Pmp has been identified herein as a conserved protein. This means that Pmp is expressed in many, if not all strains of *S. pneumoniae*. Pmp has been shown to have surface exposure and to elicit opsonophagocytic activity. These characteristics of Pmp enable the use of this protein and protein fragments or functional equivalents

thereof in the preparation of the vaccine in such manner that the vaccine can be used against nearly all strains of *S. pneumoniae*. As Pmp is depicted as the protease maturation protein *of S. pneumoniae* and as this protein has a function as a protease activator, it is therefor easily envisaged that the protease activator proteins of other bacterial species, especially of the genus *Streptococcus*, will fulfil a major role in pathogenesis. Similar protease activators from other species, for instance from *Meisseria*, can likewise be used in vaccine preparations. These homologues and functional homologues of Pmp can thus be used in the preparation of a vaccine for other bacterial species than *S. pneumoniae*. The present invention therefor also encompasses the homologues and functional homologue equivalent proteins of Pmp and fragments and their use in vaccine preparations.

In a preferred embodiment of the invention the protein or fragments thereof used in the preparation of the vaccine, is the Pmp or a (functional) homologous fragment thereof of *S. pneumoniae* strain FT231 or strain EF 3296.

It is likewise possible to employ a fragment of Pmp for the preparation of a vaccine. A fragment is a polypeptide with an amino acid sequence which is functionally similar to the corresponding section of the protein. In principle any fragment of Pmp can be used. A preferred fragment is an oligopeptide that contains one of the characterising parts or active domains of the protein. The fragment of Pmp can be (part of) an anchoring fragment, an antigenic fragment or a fragment that is (part of) a receptor binding site or an antibody binding site or combinations thereof. The Pmp or the fragment or the functional equivalent thereof can be obtained by recombinant techniques or by chemical synthesis of Pmp oligopeptides. Synthetic oligopeptides based on or derived from Pmp can for instance be obtained by conventional pepscan technology. The use of Pmp or a (homologous) fragment or a (homologous) functional equivalent thereof as a carrier in other vaccines is also encompassed by the invention. When Pmp expresses certain strongly immunogenic properties, these properties can be used by employing Pmp as a carrier. Pmp then serves to induce an immune response to a bad immunogen such as a protein or a sugar of other bacterial or viral pathogens. This strategy is useful in conjugate vaccine strategies. In an embodiment the protease maturation protein or (homologous) fragment or (homologous) functional equivalent thereof is used as a carrier protein, preferably in a conjugate vaccine strategy.

In a preferred embodiment of the invention, the fragment is an anchoring fragment, an antigenic fragment or a functional equivalent fragment thereof or a functional equivalent for a receptor binding site or an antibody binding site.

A fragment of Pmp in general will consists of an oligopeptide of at least 5 amino acids, preferably at least about 8, but oligopeptides with 10-15 amino acids are preferred. These fragments can also be used in the from of tandem oligopeptides or dimerised oligopeptides.

The protein or (functional) fragment that is used in the preparation of the vaccine can be a partially purified protein, a purified protein or fragment of Pmp.

In order to obtain a vaccine that can be administered, the protein is brought into a form that is suitable for this purpose. To this end, the protein can be conjugated with a carrier protein. Carrier proteins that can be used in this invention are in general conventional carriers and as such are well known in the art. The vaccine can likewise also comprise adjuvants and other additional components to further ensure the proper functioning of the vaccine. These additional components are generally known by the skilled man.

In a preferred embodiment of the invention, the composition comprising the protein or the fragment is therefore combined with an adjuvant and/or a carrier. From this composition a vaccine is prepared which is used in the preventive vaccination against *S. pneumoniae*. A more preferred embodiment of the invention comprises protease maturation protein of *S. pneumoniae* or a fragment thereof for the preparation of a vaccine for the preventive treatment of a *S. pneumoniae* infection.

The invention further provides for a method for the preparation of a vaccine against S. pneumoniae. The method comprises the steps of preparing or isolating the protein or the fragment or homologue or functional homologue of the protein or fragment, determining the immunogenic response by raising antibodies against the protein or the fragment or homologue or functional homologue of the protein or fragment and testing the antibodies for activity. The method according to the invention also encompasses the recombinant or synthetic production of the protein or the fragment or homologue or functional homologue of the protein or fragment and the subsequent steps to the preparation of the vaccine.

In general, in this invention, when a protein or a fragment thereof is described, the protein and the fragment encompass the Pmp of *S. pneumoniae* or a

fragment thereof, a homologous protein or fragment or a homologous or functional homologous protein or fragment thereof.

A preferred embodiment of the invention is a method for the preparation of a vaccine against *S. pneumoniae* comprising the steps of :

a. obtaining a protease maturation protein of *S. pneumoniae* or a fragment thereof or homologous or functionally homologous protein or fragment thereof; and

b. combining the protein or the fragment obtained under (a) with a suitable carrier or adjuvant.

The invention further provides a method for the vaccination of a mammal against an infection of *S. pneumoniae* comprising administering a suitable dose of the vaccine of the invention. The vaccine is suitable for vaccination against all strains and subspecies of *S. pneumoniae,* also for veterinary purposes.

The invention provides for the use of homologous Pmp proteins or fragments thereof of other *S. pneumoniae* species with amino acid sequences or fragments thereof such as peptides that are functionally homologous to the sequence depicted in fig 1B. Said functional homologous peptides can be used in a vaccine for the treatment, preferably the preventive treatment of a wide variety of strains and (sub)species of *S. pneumoniae.*

In one aspect of the invention the antibodies raised against the protein of the present invention may also provide for neutralising effects. These antibodies do not raise any opsonophagocytic activity against *S. pneumoniae* or only to a reduced extent These antibodies merely block certain epitopes of the antigen (in this case Pmp) and may disturb secretion, protection or activation of proteins, directly or indirectly involved in pneumococcal pathogenesis aspects, including colonisation and other processes of *S. pneumoniae.* This provides for an alternative way of treating *S. pneumoniae* infections.

The sequence of the *S. pneumoniae* nucleotides 820800-821738 on contig 3836 (previously known as 7632-8597 on contig 33) and the encoding polypeptide sequence harbouring Pmp are known. The nucleic acid sequence can be used to encode for Pmp or a fragment thereof. By incorporating this sequence or part thereof in a suitable vector and expressing that vector in a cell, it is possible and within the scope of the

invention to obtain recombinant peptide sequences which can subsequently be used in the preparation of a vaccine.

Accordingly the invention also relates to the use of the nucleic acid sequence or fragment thereof or a (functionally) homologous sequence or fragment thereof encoding for Pmp or a fragment thereof. The invention also provides a method for the preparation of a vaccine against *S. pneumoniae*. The method comprises the principal steps of isolating the Pmp protein or the fragment thereof, determining the immunogenic response by raising antibodies against the protein or the fragment, and testing the antibodies in *S. pneumoniae* strains. The invention also provides the recombinant protein or fragment thereof, that has been obtained, for instance, through the expression of a gene sequence encoding for the protein in a suitable vector. The invention also provides for a method of obtaining an antibody and to the antibody. An embodiment of the invention is therefor a method for obtaining an antibody against protease maturation protein comprising the steps of isolating a protease maturation protein or a fragment thereof, raising antibodies against the protein or fragment thereof and isolating the antibodies. The protein or fragment that is used in the preparation of the vaccine or in obtaining the antibody can be a recombinant or synthetic protein or fragment of Pmp.

In an embodiment of the invention, the vaccine can also be derived from the expression of recombinant nucleic acids. The Pmp gene of *S. pneumoniae* can suitably be expressed in *E. coli*.

Pmp and derivatives such as fragments for instance in the form of oligopeptides and modified oligopeptides are tested in animal models to elicit the protection against the different forms of infection (otitis media, pneumonia, sepsis, meningitis) and colonisation.

The production of Pmp for vaccine purposes is in a recombinant form wherein the gene encoding for Pmp is overexpressed in gram positive and/or gram negative bacteria. This yields Pmp in bulk quantities after which further necessary steps such as purification follow.

The present invention further pertains to a method for the identification of proteins expressing opsonophagocytic activity comprising extraction of, preferably surface associated, proteins, subjecting the obtained proteins to protein

electrophoresis, preferably 2D, obtaining antisera against the proteins, and subjecting the antibodies to an opsonophagocytic assay.

The method provides for a rapid and efficient screening of a large number of proteins or fragments thereof and allows for the rapid identification of proteins of interest. The method according to this aspect of the invention is surprising in that the combination protein electrophoresis and an opsonophagocytic assay results in proteins that are considered to have immunoprotective properties. Electrophoresis techniques use denatured proteins. Antibodies that are active in opsonophagocytic assays are preferably directed to epitopes of the native protein. It is a surprising aspect of the present invention that by the combination of these two methods, antibodies are obtained that allow for immunoprotective properties.

Alternatives for the opsonophagocytic assay are *in vivo* passive immunoprotection assay, *in vivo* active immunoprotective assay and *in vivo* active immunoprotective assay. These techniques are by itself well known in the art and may also serve to identify vaccine candidates according to the invention. By varying the extraction techniques, for instance by varying the detergent or by using chromatographic techniques such as column chromatography, protein fractions of varying composition can be isolated which can be further processed according to the method. It is likewise possible to directly identify the proteins after the electrophoresis step, prior to assaying the proteins for instance by using mass-spectroscopic techniques such as Maldi-tof.

Description of the Figures:

Figure 1 : the *S. pneumoniae* nucleotides 820800-821738 on contig 3836 (http://www.tigr.org/data/*S.pneumoniae* /) (A) and the encoding polypeptide sequence (B) harbouring Pmp. The presumed methionine start codon of Pmp is depicted in bold and underscored.

Figure 2 : The protease maturation protein of *Lactobacillus paracasei* (Swiss Prot acc. nr. Q02473).

Figure 3 : The protease maturation protein of *Lactococcus lactis subspec. lactis* (Swiss Prot acc. nr. P15294)

Figure 4 : The protease maturation protein of *Lactococcus lactis subsp. cremoris* (Swiss Prot acc. nr. P14308)

MATERIALS AND METHODS

Extraction of surface-associated, hydrophobic proteins of *S. pneumoniae*.

S. pneumoniae FT231 and *S. pneumoniae* EF3296 were cultured at 37 °C in Todd Hewitt broth (Difco laboratories, Detroit, USA) supplemented with 0.5% Yeast Extract (Difco laboratories). At logarithmic growth phase (OD_{550}=0.3) the bacteria were harvested by centrifugation, and washed three times with phosphate-buffered saline pH 7.5 (PBS). After the final washing the bacteria were resuspended in TE-buffer (10 mM Tris-Cl, 1 mM EDTA). The cells were disrupted by ultrasonic treatment (Branson sonifier 250, Branson Ultrasonics, Danburry, USA).

Extraction with sulfobetaïne 14 (SB14) was performed as described by Schouls *et al.* (22). In brief, the water-soluble cytoplasmic proteins were removed by washing the bacterial lysates five times with PBS. Cell walls, membranes and other particulate material were collected by centrifugation at 48,400*g for 20 min. Pellets were resuspended in 150 mM NaCl and centrifuged for 20 min at 48,400*g. The pellets were then incubated for 2 hours at room temperature with 0.25% N-tetradecyl-N,N-dimetylammonio-1-propanesulfonate (SB14, Serva, Heidelberg, Germany) in the presence of 150 mM NaCl, 10 mM $MgCl_2$ and 10 mM Tris-HCl pH 8.0 during constant stirring. The hydrophobic, membrane-associated proteins were recovered as described by Wessel and Flügge (23).

Extraction with Triton X114 (Sigma, St. Louis, USA) was also performed as described by Schouls *et al.* (24). Briefly, bacterial lysates were centrifuged at 20,000*g for 20 min. Pellets were dissolved with 1% Triton X114 in PBS for 1 hour at 0 °C.

After extraction, the suspensions were centrifuged at 25,000* g at 4 °C for 1 hour, the supernatants were incubated at 37 °C for 30 min, and centrifuged at 25,000*g at 25 °C for 1 hour to separate the detergent phase and aqueous phase. The proteins in the detergent phase were extracted according to the procedure of Wessel and Flügge (23). Protein concentrations were measured by the method of Bradford (25).

Protein electrophoresis and staining.

One-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in the Biorad minigel system with 13% polyacrylamide gels. The samples were dissolved in sample buffer (10 mM Tris-HCl, 1 mM EDTA, 1% SDS, 10 mM DTT, 1% glycerol, 0.01% bromophenol blue indicator (Merck, Darmstadt, Germany), boiled for 5 min and subjected to electrophoresis (26).

Two-dimensional SDS-PAGE was performed according to the instructions of the manufacturer (Pharmacia Biotech, Uppsala, Sweden) including modifications of Rabilloud *et al.* (27). After isoelectric focusing, proteins were separated using gradient (12-20%) polyacrylamide gel electrophoresis.

Silver staining of polyacrylamide gels was performed as described by Blum *et al.* (28). In addition, standard procedures were used to stain the polyacrylamide gels using Coomassie brilliant blue (CBB) (26).

The software program PD Quest (PDI, New York, USA) was used for the computerised analysis of two-dimensional SDS-PAGE gels.

Hyperimmune rabbit antiserum.

Hyperimmune antiserum was raised against the hydrophobic, surface-associated proteins by injecting New-Zealand White rabbits subcutaneously into 4-5 places. The SB14 and Triton X114-extracted hydrophobic surface-associated proteins (500 µg) of *S. pneumoniae* FT231 and EF3296, respectively, were dissolved in 0.5 ml 0.9% NaCl, and subsequently mixed with 0.5 ml Freund's incomplete adjuvant (Pierce, Rockford, USA). In addition, hyperimmune rabbit serum was raised against SB14-purified hydrophobic surface-associated proteins of *S. pneumoniae* FT231 that

were subjected to 1D-SDS-PAGE. The total protein pool was cut from the polyacrylamide gel, washed three times with 0.1 M NaAc, 96% EtOH, ground into a fine suspension in 0.5 ml PBS, and subsequently mixed with 0.5 ml Freund's incomplete adjuvant. Negative control serum was gained by injection of washed and ground polyacrylamide in 0.5 ml PBS mixed with 0.5 ml Freund's incomplete adjuvant. The primary injection was followed by four subcutaneous booster injections at four-week intervals.

Antibodies to type 2 capsule were purchased from Statens Seruminstitut, Copenhagen, Denmark. Recombinant pneumolysin was used to raise hyperimmune sera in rabbits as described previously (29). These sera were used as positive controls in passive immunisation experiments.

Indirect immuno-cytometric assay.

Pneumococci were grown to logarithmic phase in Todd-Hewitt broth supplemented with 0.5% Yeast Extract at 37 °C using 5% CO_2, then washed three times in ice-cold PBS and stored overnight at 4 °C. The bacteria were incubated in 5% rabbit serum (10^7 bacteria in 20 µl final volume) for 15 min at 4 °C while shaking. The bacteria were washed twice using ice-cold PBS and incubated for 15 min at 4 °C with 20 µl (1:5 dilution) of fluorescein-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, USA) while shaking. The bacteria were washed twice with ice-cold PBS and resuspended in 100 µl of ice-cold paraformaldehyde (0.5 %) in PBS. The samples were analysed in a FACScan flow cytometer (Becton Dickinson, Mountain View, USA).

Phagocytosis assay.

Analysis of the opsonophagocytic activity of the sera was performed as described by Alonso Develasco *et al.* (30). In brief, *S. pneumoniae* was grown to logarithmic phase in Todd-Hewitt broth supplemented with 0.5% Yeast Extract at 37 °C using 5% CO_2. After washing with PBS, the bacteria were labeled with fluorescein-isothiocyanate (FITC, isomer I, Sigma Chemical Co., St. Louis, USA) (0.5 mg/ml in

PBS) for 1 hour at 4 °C, washed twice and resuspended in Hank's balanced salt solution (HBSS) containing 1% w/v bovine BSA. The bacteria (10^8 bacteria per 100 µl BSA-HBSS) were stored at -20 °C. Samples of 2.5 * 10^6 bacteria were transferred into round-bottom microtiter plates (Greiner Labortechnik, Alphen a/d Rijn, The Netherlands). Rabbit sera diluted in BSA-HBSS and heat-inactivated for 30 min at 56 °C were added to the bacteria (final volume 50 µl). The opsonisation was performed at 37 °C for 30 min while shaking. Plates were then placed on ice and 2.5 * 10^5 human polymorphonuclear cells isolated from peripheral blood of healthy volunteers were added to each well (final volume 100 µl). Human PMNs were isolated by mixing 30 ml of heparinised blood with 30 ml of phosphate-buffered saline (pH 7.4), layered on Ficoll-Paque, and centrifuged for 20 min at 400*g. The lowest layer containing PMNs and erythrocytes was washed once in RPMI (Gibco BRL, Life Technologies LTD, Paisley, UK) containing 0,05% human serum albumin. The erythrocytes were lysed using ice-cold lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA, pH 7.4). Phagocytosis was performed for 30 min at 37 °C while shaking. After washing twice with ice-cold HBSS, samples were resuspended in 200 µl of HBSS. The PMNs were fixed by adding 100 µl PBS-2% paraformaldehyde, and the samples were analysed in a FACScan flow cytometer (Becton Dickinson). Fluorescent PMNs observed after opsonisation with antiserum indicates both uptake and binding (referred to as phagocytosis) of FITC-labelled bacteria. The opsonophagocytic activity is defined as the reciprocal of the serum concentration at which 25 % of the human PMNs were fluorescent.

Immuno electron microscopy.

Immuno electron microscopy was performed according to the standard operational procedures of the national institute for biological standards and control, Potters bar, United Kingdom.

Purification, tryptic digest and mass spectrometric analysis of the proteins.

The protein gel spots of interest were excised from the gel. The gel fragments were sliced thinly and washed twice for 15 minutes in 5 % trichloro acetic acid

($C_2HCl_3O_2$; Merck, Darmstadt, Germany) and three times in distilled water. The gel fragments were equilibrated in sample buffer pH 6.8 (0.1 % SDS, 10 % glycerol, 50 mM DTT, 12 mM Tris-HCl, 0.01 % bromophenol-blue) for 1 hour at room temperature.

The proteins were concentrated by an agarose electrophoresis (1 % agarose type VIII, Sigma, St. Louis, USA) method as described by Rider et al. (Rider, M. H., M. Puype, J. van Damme, K. Gevaert, S. de Boeck, J. D'Alayer, H. H. Rasmussen, J. E. Celis, and J. Vanderkerckhove. 1995. An agarose-based gel-concentration system for microsequence and mass spectrometric characterization of proteins previously purified in polyacrylamide gels starting at low picomole levels. Eur. J. Biochem. 230:258-265.) and Gevaert et al. (Gevaert, K., J. Verschelde, M. Puype, J. van Damme, M. Goethals, S. de Boëck, and J. Vanderkerckhove. 1996. Structural analysis and identification of gel-purified proteins in the femtomole range, using a novel computer program for peptide sequence assignment, by matrix-assisted laser desorption ionisation-reflection time-of-flight-mass spectrometry. Electrophoresis. 17:918-924) on a Bio-Rad model 150-A gel electrophoresis cell (Bio-Rad laboratories, Richmond, USA) with Pasteur pipettes. After staining the agarose gel with carconcarboxylic acid (Sigma), the proteins were excised from the gel. The agarose fragments were washed with distilled water, and resuspended in 18 µl of digestion buffer pH 8.0 (50 mM NH_4HCO_3, 5 mM $CaCl_2$). The agarose was melted at 85 °C for 1 minute. After cooling down to 37 °C 0.05 µg/µl trypsin (trypsin modified sequencing grade, Promega, Madison, USA) was added to digest the proteins for at least 15 hours at 37 °C. Trypsin was inactivated by adding 1 µl of 10 % trifluoro acetic acid ($C_2HF_3O_2$; Merck).

The tryptic digests were analysed using a reversed phase micro-capillary column switching HPLC system (Meiring, H. D., B. M. Barroso, E. van der Heeft, G. J. ten Hove, and A. P. J. M. de Jong. 1999. Sheathless *Nano*flow HPLC-ESI/MS(n) in Proteome Research and MHC Bound Peptide Identification. In Proceedings of the 47th ASMS Conference on Mass Spectrometry and Allied Topics, Dallas, Texas.; van der Heeft, E., G. J. ten Hove, C. A. Herberts, H. D. Meiring, C. A. C. M. van Els, and A. P. J. M. de Jong. 1998. A microcapillary column switching system HPLC-electrospray ionisation MS system for the direct identification of peptides presented by major histocompatibility complex class I molecules. Anal. Chem. 70:3742-3751.).

Peptide sequencing was performed on a LCQ quadropole ion trap mass spectrometer (Finnigan MAT, San Jose, CA, USA). Tandem mass spectrometric data were collected in data dependent scan mode for sequence information of single tryptic digest products. With Peptide Search (Mann, M., and M. Wilm. 1994. Error-tolerant identification of peptides in sequence databases by peptide sequence tags. Anal. Chem. 66:4390-4399.), the deduced (partial) amino acid sequences were analysed for matching sequences in all possible translation products of the most current version of the unfinished pneumococcal genome released by The Institute for Genomic Research (TIGR; http://www.tigr.org/data/s_pneumoniae/) to identify the proteins. With the BLAST algorithm (Altschul, S. F., G. W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. 251:403-410), putative pneumococcal proteins were analysed for similarity to sequences deposited in the November 1999 version of the non-redundant protein database at the National Center for Biotechnology Information (Washington D.C., USA).

Further proof of principle can be obtained by immunisation experiments in various animal models (mice, rats, rabbits) using purified Pmp, recombinant Pmp or derivatives ad fragments of Pmp.

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Amended claims

1. A vaccine or medical preparation comprising a protease maturation protein of *S. pneumoniae* comprising an amino acid sequence as shown in fig 1B, and/or a fragment thereof and/or a homologous and/or a functionally homologous protein or protein fragment thereof for the treatment of microbial infections.

2. The vaccine or medical preparation according to claim 1 for the treatment of *S. pneumoniae*.

3. The vaccine or medical preparation according to claim 1 or 2, further comprising a suitable adjuvant or carrier.

4 The vaccine or medical preparation according to anyone of the claims 1-3 wherein said protein is the protein maturation protein from *S. pneumoniae* Ft231 or EF3296.

5. The vaccine or medical preparation according to anyone of the claims 1-4 wherein said fragment comprises an anchoring fragment, an antigenic fragment or a functional equivalent thereof or a functional equivalent of a receptor binding site or an antibody binding site.

6 The vaccine or medical preparation according to anyone of the claims 1-5 wherein said protein or said fragment comprises a purified, recombinant or synthetic protein or fragment thereof.

7. The vaccine or medical preparation according to anyone of the claims 1-6 wherein said fragment comprises at least 8 amino acids.

8. Method for the preparation of a vaccine against *S. pneumoniae* comprising the steps of:

a. isolating a protease maturation protein of *S. pneumoniae* comprising an amino acid sequence as shown in fig 1B, or a fragment thereof or a recombinant or synthetic protein or fragment thereof or homologous or functionally homologous protein or fragment thereof; and

b. combining the protein or the fragment thereof obtained under (a) with a suitable carrier or adjuvant.

9. Method for obtaining an antibody against the protease maturation protein of *S. pneumoniae*, the method comprising the steps of isolating protease maturation protein comprising an amino acid sequence as shown in fig 1B or a fragment thereof and/or a homologous and/or a functionally homologous protein or protein fragment thereof, and raising antibodies against said protein or fragment thereof.

10. Antibody comprising opsonophagocytic activity obtainable by the method according to claim 9.

11. Use of a protease maturation protein of *S. pneumoniae* comprising an amino acid sequence as shown in fig 1B, or a fragment thereof and/or a homologous and/or a functionally homologous protein or protein fragment thereof, for the preparation of a vaccine for the treatment or prophylaxis of a *S. pneumoniae* infection.

12. Use of a protease maturation protein of *S. pneumoniae* comprising an amino acid sequence as shown in fig 1B, or a fragment thereof or a recombinant or synthetic protein or fragment thereof as a carrier.

13. Method of treatment of a *S. pneumoniae* infection comprising administering a vaccine according to claims 1-7.

14. Method for the vaccination of a mammal against an infection of *S. pneumoniae* comprising administering a suitable dose of a vaccine according to anyone of the claims 1-7.

15. Use of a nucleic acid sequence coding for a protease maturation protein comprising an amino acid sequence as shown in fig 1B, or a fragment thereof and/or a homologous and/or a functionally homologous protein or protein fragment thereof, for obtaining a recombinant protease maturation protein or fragment thereof.

16. Cell containing a recombinant nucleic acid sequence or a vector encoding for protease maturation protein comprising an amino acid sequence as shown in fig 1B, or a fragment thereof and/or a homologous and/or a functionally homologous protein or protein fragment thereof.

17. Recombinant protease maturation protein comprising an amino acid sequence as shown in fig 1B, or fragment thereof and/or a homologous and/or a functionally homologous protein or protein fragment thereof, obtainable through the expression of a gene sequence encoding for said protein in a suitable vector.

18. Use of protease maturation protein of *S. pneumoniae* comprising an amino acid sequence as shown in fig 1B, and/or a fragment thereof and/or a homologous and/or a

functionally homologous protein or protein fragment thereof for the preparation of a medicament for the treatment of diseases connected with *S. pneumoniae* infections.

19. Use of protease maturation protein of *S. pneumoniae* comprising an amino acid sequence as shown in fig 1B, and/or a fragment thereof and/or a homologous and/or a functionally homologous protein or protein fragment thereof for eliciting opsonophagocytic activity and/or in vivo immunisation and/or in vivo immune protection against *S. pneumoniae*.

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(54) Title: PNEUMOCOCCAL VACCINES

(57) Abstract: The invention relates to the use of a protein or a fragment thereof of *S. pneumoniae*, its use for the preparation of a vaccine for the preventive treatment of a *S. pneumoniae* infection, compositions comprising protease maturation protein of *S. pneumoniae* infection, or a fragment thereof, vaccines comprising said protein or fragment thereof, use of a nucleic acid sequence encoding for said protein or fragment thereof, vectors wherein the nucleic acid sequence is brought to expression and to recombinant protease maturation protein or a fragment thereof or (functional) homologues thereof and to a method for the determination of proteins with opsonophagocytic activity and/or *in vivo* immunisation and/or *in vivo* immune protection.

Figure 1.

Fig 1A.

AGTAACACTTATCTCAAAGGAGTAGACATGAAGAAAAAATTATTGGCAGGTG
CCATCACACTATTATCAGTAGCAACTTTAGCAGCTTGTTCGAAAGGGTCAGAAGGTG
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AA

Fig 1B.

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AGMTLETRKAQIRTSKLVELAVKKVAEAELTDEAYKKAFDEYTPDVTAQIIRLNNED
KAKEVLEKAKAEGADFAQLAKDNSTDEKTKENGGEITFDSASTEVPEQVKKAAFALD
VDGVSDVITATGTQAYSSQYYIVKLTKKTEKSSNIDDYKEKLKTVILTQKQNDSTFV
QSIIGKELQAANIKVKDQAFQNIFTQYIGGGDSSSSSSTSNE

Figure 2

mkkkmrlkvllastatallllsgcqsnqadqkvatysggkvtesnfykelkq
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sfkeslrtnflsevalkklkkvsesqlkavwktyqpkvtvqhiltsdedtakqvisd
laagkdfatlaktdsidtatkdnggkisfesnnktldatfkdaayklkngdytqtpv
kvtngyevikminhpakgtftsskkaltasvyakwsrdssimqrvisqvlknqhvti
kdkdladaldsykkpattn

Figure 3

mkkkmrlkvllastatallllsgcqsnqtdqtvatysggkvtessfykelkq
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kdkdladaldsykklattn

Figure 4

mkkkmrlkvllastatallllsgcqsnqtdqtvatysggkvtesslykelkq
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kvtdgyevikminhpakgtftsskkaltasvyakwsrdssimqrvisqvlknqhvti
kdkdladaldsykklattn

Declaration and Power of Attorney Patent Application (Design or Utility)

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: 'Pneumococcal vaccines'

the specification of which

☐ is attached hereto

x was filed on February 12, 2002 as application serial no. 10/049,473 and or PCT International Application number PCT/NL00/00569 and was amended on (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information know to me to be material to patentability as defined in 37 C.F.R.§1.56.

I hereby claim foreign priority benefits under 35 U.S.C.§119(a)-(d) or 35 U.S.C.§365(b) of any foreign application(s) for patent or inventor's certificate, or 35 U.S.C.§365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below any foreign application for patent or inventor's certificate of PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)		
Number 99202640.1	**Country** EP	**Day/Month/Year Filed** 13-08-1999
Number	**Country**	**Day/Month/Year Filed**
Number	**Country**	**Day/Month/Year Filed**

10049473 .073002

Power of Attorney

As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

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Glenn T. Henneberger	36,074
Richard LaCava	41,135
Kevin E. McDermott	35,946
Robert C. Morriss	42,910
Samir R. Patel	P-44,998
R. Glenn Schroeder	34,720
Susan A. Sipos	43,128
Roderick S.W. Turner	38,639
Steven T. Zuschlag	43,309

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I hereby authorize them or others whom they may appoint to act and rely on instructions from and communicate directly with the person/organization who/which first sends this case to them and by whom/which I hereby declare that I have consented after full disclosure to be represented unless/until I instructed otherwise.

Please direct all correspondence in this case to at the address indicated below:

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Residence and Citizenship		
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Post Office Address		
Street Address	**City**	**State & Zip Code or Country**
Jan van Ghestellaan 45	Rotterdam	3054 CG 3054
Signature of Inventor		**Date** 19-02-2002

1 of 2

10/049473 .073002

2-W

Full Name of Second Inventor, if any		
Family Name Hermans	**First Given Name** Peter	**Second Given Name** Wilhelmus, M.
Residence and Citizenship		
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Street Address Bachlaan 204	**City** Oud-Beijerland-gerland	**State & Zip Code or Country** 3261 WO
Signature of Inventor		**Date** 19/II/02

SEQUENCE LISTING

<110> de Groot, Ronald
Hermans, Peter Wilhelmus Maria

<120> Pneumococcal Vaccines

<130> Docket 294-120 PCT/US

<140> US 10/049,473
<141> 2002-02-12

<150> PCT/NL00/00569
<151> 2000-08-14

<150> EP 99202640.1
<151> 1999-08-13

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294-120PCT-US sequence listing

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Val Leu Leu Asn Met Thr Ile Gln Lys Val Phe Glu Lys Gln Tyr Gly
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Lys Gln Tyr Gly Glu Asn Tyr Gln Arg Val Leu Ser Gln Ala Gly Met
            100                 105                 110

Thr Leu Glu Thr Arg Lys Ala Gln Ile Arg Thr Ser Lys Leu Val Glu
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Glu Lys Thr Lys Glu Asn Gly Gly Glu Ile Thr Phe Asp Ser Ala Ser
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Asp Gly Val Ser Asp Val Ile Thr Ala Thr Gly Thr Gln Ala Tyr Ser
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275 280 285

Gln Ala Ala Asn Ile Lys Val Lys Asp Gln Ala Phe Gln Asn Ile Phe
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Thr Gln Tyr Ile Gly Gly Gly Asp Ser Ser Ser Ser Ser Ser Thr Ser
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Asn Glu

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<213> Lactobacillus paracasei

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Glu Leu Lys Gln Ser Pro Thr Thr Lys Thr Met Leu Ala Asn Met Leu
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Ile Tyr Arg Ala Leu Asn His Ala Tyr Gly Lys Ser Val Ser Thr Lys
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Thr Val Asn Asp Ala Tyr Asp Ser Tyr Lys Gln Gln Tyr Gly Glu Asn
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Phe Asp Ala Phe Leu Ser Gln Asn Gly Phe Ser Arg Ser Ser Phe Lys
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Pro Lys Val Thr Val Gln His Ile Leu Thr Ser Asp Glu Asp Thr Ala
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Lys Gln Val Ile Ser Asp Leu Ala Ala Gly Lys Asp Phe Ala Thr Leu
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Ile Ser Phe Glu Ser Asn Asn Lys Thr Leu Asp Ala Thr Phe Lys Asp
195 200 205

Ala Ala Tyr Lys Leu Lys Asn Gly Asp Tyr Thr Gln Thr Pro Val Lys
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Val Thr Asn Gly Tyr Glu Val Ile Lys Met Ile Asn His Pro Ala Lys
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Gly Thr Phe Thr Ser Ser Lys Lys Ala Leu Thr Ala Ser Val Tyr Ala
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<213> Lactococcus lactis lactis

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20 25 30

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35 40 45

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294-120PCT-US sequence listing

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85 90 95

Phe Asp Ala Phe Leu Ser Gln Asn Gly Phe Ser Arg Ser Ser Phe Lys
100 105 110

Glu Ser Leu Arg Thr Asn Phe Leu Ser Glu Val Ala Leu Lys Lys Leu
115 120 125

Lys Lys Val Ser Glu Ser Gln Leu Lys Ala Ala Trp Lys Thr Tyr Gln
130 135 140

Pro Lys Val Thr Val Gln His Ile Leu Thr Ser Asp Glu Asp Thr Ala
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Lys Gln Val Ile Ser Asp Leu Ala Ala Gly Lys Asp Phe Ala Met Leu
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Ala Lys Thr Asp Ser Ile Asp Thr Ala Thr Lys Asp Asn Gly Gly Lys
180 185 190

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195 200 205

Ala Ala Tyr Lys Leu Lys Asn Gly Asp Tyr Thr Gln Thr Pro Val Lys
210 215 220

Val Thr Asp Gly Tyr Glu Val Ile Lys Met Ile Asn His Pro Ala Lys
225 230 235 240

Gly Thr Phe Thr Ser Ser Lys Lys Ala Leu Thr Ala Ser Val Tyr Ala
245 250 255

Lys Trp Ser Arg Asp Ser Ser Ile Met Gln Arg Val Ile Ser Gln Val
260 265 270

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Leu Asp Ser Tyr Lys Lys Leu Ala Thr Thr Asn
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294-120PCT-US sequence listing

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Val Ala Thr Tyr Ser Gly Gly Lys Val Thr Glu Ser Ser Leu Tyr Lys
        35                  40                  45

Glu Leu Lys Gln Ser Pro Thr Thr Lys Thr Met Leu Ala Asn Met Leu
    50                  55                  60

Ile Tyr Arg Ala Leu Asn His Ala Tyr Gly Lys Ser Val Ser Thr Lys
65                  70                  75                  80

Thr Val Asn Asp Ala Tyr Asp Ser Tyr Lys Gln Gln Tyr Gly Glu Asn
                85                  90                  95

Phe Asp Ala Phe Leu Ser Gln Asn Gly Phe Ser Arg Ser Ser Phe Lys
            100                 105                 110

Glu Ser Leu Arg Thr Asn Phe Leu Ser Glu Val Ala Leu Lys Lys Leu
        115                 120                 125

Lys Lys Val Ser Glu Ser Gln Leu Lys Ala Ala Trp Lys Thr Tyr Gln
    130                 135                 140

Pro Lys Val Thr Val Gln His Ile Leu Thr Ser Asp Glu Asp Thr Ala
145                 150                 155                 160

Lys Gln Val Ile Ser Asp Leu Ala Ala Gly Lys Asp Phe Ala Met Leu
                165                 170                 175

Ala Lys Thr Asp Ser Ile Asp Thr Ala Thr Lys Asp Asn Gly Gly Lys
            180                 185                 190

Ile Ser Phe Glu Leu Asn Asn Lys Thr Leu Asp Ala Thr Phe Lys Asp
        195                 200                 205

Ala Ala Tyr Lys Leu Lys Asn Gly Asp Tyr Thr Gln Thr Pro Val Lys
    210                 215                 220

Val Thr Asp Gly Tyr Glu Val Ile Lys Met Ile Asn His Pro Ala Lys
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225 230 235 240

Gly Thr Phe Thr Ser Ser Lys Lys Ala Leu Thr Ala Ser Val Tyr Ala
245 250 255

Lys Trp Ser Arg Asp Ser Ser Ile Met Gln Arg Val Ile Ser Gln Val
260 265 270

Leu Lys Asn Gln His Val Thr Ile Lys Asp Lys Asp Leu Ala Asp Ala
275 280 285

Leu Asp Ser Tyr Lys Lys Leu Ala Thr Thr Asn
290 295